FINAL SCRIPT: APPROVED FOR FILMING

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Title: Determination of Mitochondrial Morphology in Live Cells Using Confocal Microscopy

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **No.**

Current Protocol Length
Number of Steps: 15
Number of Shots: 31

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Manna Lin:** Our research focuses on basic neuropharmacology. Specifically, we study how drug molecules work. We aim to understand the relationship between potential drug compounds and mitochondrial function in Parkinson's disease **[1]**.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What research gap are you addressing with your protocol?

1.2. **Manna Lin:** Prior studies reveal long-standing live-cell analysis challenges even with manufacturer guidelines. To solve this, we outline a detailed, step-by-step protocol for assessing mitochondrial morphology in live cells **[1]**.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B. roll: 4.2*

What advantage does your protocol offer compared to other techniques?

1.3. **Jiakang Wang:** Our protocol uses a simple mitochondrial stain, fluorescence imaging, and open-source software. This reduces costs, increases accessibility, and makes it easier to use than complex alternatives like electron microscopy **[1]**.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B. roll: 2.7*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. MPP+ Stimulation and MitoTracker Staining of SH-SY5Y Cells

Demonstrator: Manna Lin

2.1. To begin, obtain cultures of SH-SY5Y *(S-H-S-Y-Five-Y)* cells grown in flasks containing DMEM/F12 (D-M-E-M-F-Twelve), supplemented with 10 percent FBS *(F-B-S)* **[1-TXT].**

2.1.1. WIDE: Talent taking out a culture flask from the humidified incubator. **TXT: DMEM/F12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12**

2.2. Detach the cells for 1 minute using a 0.05 percent trypsin solution **[1].** Then, centrifuge the cells at 100 g for 3 minutes before passaging **[2]**.

2.2.1. Talent adding trypsin solution to the flask.

2.2.2. Talent placing the tube in a centrifuge.

2.3. Next, suspend the SH-SY5Y cells in DMEM/F12 medium supplemented with 1% FBS **[1].** Then seed the cells onto confocal dishes with glass bottoms and maintain them overnight **[2].**

2.3.1. Talent suspend SH-SY5Y cells in DMEM/F12.

2.3.2. Talent seeding the cells into confocal dishes.

2.4. For MPP+ *(M-P-P-Plus)* stimulation, replace the culture medium with fresh medium **[1-TXT]** with or without 1-methyl-4-phenylpyridinium for 24 hours **[2]**.

2.4.1. Talent aspirating the old medium. **TXT: MPP+: 1-methyl-4-phenylpyridinium iodide** Videographer's Note: This is 2.4.1 to 2.4.2 (1)

2.4.2. Talent adding MPP+ solution and placing the dishes back in the incubator. Videographer's Note: This is 2.4.1 to 2.4.2 (2)

2.5. For mitoTracker *(my-toe-tracker)* staining, dilute MitoTracker stock solution with prewarmed DMEM-F12 medium to obtain a 50-nanomolar working concentration **[1].** Keep the staining solution protected from light throughout the procedure **[2].**

2.5.1. Talent pipetting and mixing the MitoTracker solution into a tube with DMEM-F12.

2.5.2. Talent covering the staining solution tube with aluminum foil.

2.6. Remove the culture medium from the confocal dishes **[1].** Wash the cells twice with fresh DMEM/F12 **[2]**.

2.6.1. Shot of the culture medium being aspirated from the confocal dishes.

2.6.2. Talent adding fresh medium to the dishes, swirly it and removing the medium.

2.7. Then pipette 1 milliliter of the MitoTracker working solution into the dish **[1].** Incubate for 15 minutes at 37 degrees Celsius in the dark **[2]**.

2.7.1. Talent adding MitoTracker solution to dishes.

2.7.2. Talent placing them in a dark incubator.

2.8. Now, remove the MitoTracker working solution **[1]** and wash the dishes twice with DMEM/F12 **[2]**. After the last wash, add 1 milliliter of DMEM/F12 to each dish **[3].** Incubate in the cell incubator before imaging **[4]**.

2.8.1. Talent aspirating the dye.

2.8.2. Shot of DMEM-F12 being added to the dish.

2.8.3. Talent adding fresh medium.

2.8.4. Talent placing dishes back into the incubator.

3. Live Imaging of Mitochondrial Dynamics in SH-SY5Y Cells

3.1. To preequilibrate the confocal microscope for imaging, under the **acquisition panel**, adjust the light path parameters by setting the excitation and emission wavelengths **[1]**. Set the **pinhole size** to 1.2 airy units, **pixel scan size** to 1,024 by 1,024, **pixel dwell time** to 2.4 microseconds with averaging of 2 **[2]**.

3.1.1. SCREEN: 68167_screenshot_3.1.1 00:00-00:16.

3.1.2. SCREEN: 68167_screenshot_3.1.2 00:00-00:14

3.2. Now, use the halogen light to focus on the cells using a plan apochromatic 60X objective lens with a numerical aperture of 1.4, minimizing phototoxicity **[1]**. Adjust the laser power and high voltage just below the saturation level, then set the zoom factor to 3 and capture the images **[2]**.

3.2.1. Talent operating the microscope.

3.2.2. SCREEN: 68167_screenshot_3.2.2 00:00-00:30.

3.3. Move the region of interest to focus on other cells under the microscope, then capture images without changing any settings **[1]**.

3.3.1. SCREEN: 68167_screenshot_3.3.1 00:10-00:35.

3.4. To enhance image quality prior to binary transformation and skeletonization, first open the image **[1].** Select an area of a single cell using the **ROI** *(R-O-I)* **tool [2]**.

3.4.1. SCREEN: 68167_screenshot_3.4.1 00:00-00:10.

3.4.2. SCREEN: 68167_screenshot_3.4.2 00:00-00:11.

3.5. Now sequentially click on **Process, Filters, Unsharp Mask** function to enhance sharpness **[1]**, then use **Process** and **Enhance Contrast** to reduce noise amplification **[2]**. Click on **Process, Filters,** and the **Median** option to remove salt and pepper noise **[3]**.

3.5.1. SCREEN: 68167_screenshot_3.5.1 00:00-00:13.

3.5.2. SCREEN: 68167_screenshot_3.5.2 00:00-00:10.

3.5.3. SCREEN: 68167_screenshot_3.5.3 00:00-00:14.

3.6. To convert the image to binary, click on **Process, followed by Binary,** and choose the **Make Binary** option **[1]**. Skeletonize the binary image by clicking **Process, Binary,** and **Skeletonize [2]**.

3.6.1. SCREEN: 68167_screenshot_3.6.1 00:00-00:10.
3.6.2. SCREEN: 68167_screenshot_3.6.2 00:00-00:10.

3.7. Then group all skeleton pixels by selecting **Analyze** followed by **Skeleton** and **Analyze Skeleton (2D/3D)** *(Skeleton-2-D-3D)* **function [1]**. Simplify the analysis by using the MiNA *(ˈmiːnə)* tool to calculate nine descriptive parameters in the skeletonized image **[2]**.

3.7.1. SCREEN: 68167_screenshot_3.7.1 00:00-00:10.

3.7.2. SCREEN: 68167_screenshot_3.7.2 00:00-00:16

Results

4. Results

4.1. The fluorescence intensity of mitochondrial staining increased progressively with MitoTracker Red CMXRos *(C-M-X-raws)* concentration from 25 nanomolar to 200 nanomolar **[1]**, but at 200 nanomolar, substantial cytoplasmic background staining was observed **[2]**.

4.1.1. LAB MEDIA: Figure 1A. *Video editor: Highlight the increasing brightness of the red signal across the four panels showing 25, 50, 100, and 200 nanomolar.*

4.1.2. LAB MEDIA: Figure 1A. *Video editor: Highlight the cell in the 200 nanomolar panel*

4.2. Mitochondrial morphology was preserved better in DMEM/F12 medium compared to PBS at 1-hour post-staining **[1]**, although DMEM/F12 also showed higher background fluorescence **[2]**.

4.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the panel labeled "DMEM/F12 1 h"*

4.2.2. LAB MEDIA: Figure 1B. *Video editor: Highlight the visibly brighter red background in the "DMEM/F12 0 h" panel compared to "PBS 0 h".*

4.3. Dual-frame averaging significantly improved the signal-to-noise ratio in mitochondrial imaging compared to single-scan mode **[1]**, and increasing pixel dwell time from 2.4 to 10.8 microseconds resulted in brighter images but caused photobleaching **[2]**.

4.3.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the top left image "average2x Dwell time2.4 Before" panel and then the top right image "average1x Dwell time2.4".*

4.3.2. LAB MEDIA: Figure 1C. *Video editor: Highlight the intensely bright signal in "average2x Dwell time10.8" and the faded signal in the "After" panel directly below it.*

4.4. Increasing high voltage intensified background noise **[1]** while increased laser power led to photobleaching **[2]**.

4.4.1. LAB MEDIA: Figure 1D. *Video editor: Sequentially highlight the images of the top row from left to right*

4.4.2. LAB MEDIA: Figure 1D. *Video editor: Sequentially highlight the images of the bottom row from left to right*

4.5. The MiNA tool produced consistent skeletonization outputs for both dim and bright images, indicating robust segmentation across different fluorescence intensities **[1]**, with the bright image exhibiting 5.5-fold higher intensity than the dim image **[2]**.

4.5.1. LAB MEDIA: Figure 1E. *Video editor: please show top row first then bottom row.*

4.5.2. LAB MEDIA: Figure 1E. *Video editor: Highlight the "Bright original image skeletonized" then the "Dim original image skeletonized".*

4.6. MPP+ treatment at 500 micromolar disrupted mitochondrial morphology in SH-SY5Y cells compared to the untreated control **[1]**, resulting in a significantly reduced mitochondrial footprint **[2]**.

4.6.1. LAB MEDIA: Figure 2A. *Video editor: Show the right side panel images (top and bottom) then show control images*

4.6.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the shorter bar labeled "MPP+ (500 µM)"*

Pronunciation Guide:

1. confocal
Pronunciation link: https://www.merriam-webster.com/dictionary/confocal merriam-webster.com+6youtube.com+6youtube.com+6
IPA: /kənˈfoʊkəl/
Phonetic Spelling: kun-FOH-kəl

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2. apochromatic
(Scientific term; not yet searched but typical pronunciation)
Pronunciation link: No confirmed link found
IPA: /ˌeɪpoʊkrəˈmætɪk/
Phonetic Spelling: ay-poh-krō-MAT-ik

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3. skeletonization
(Complex noun form of "skeletonize")
Pronunciation link: No confirmed link found
IPA: /ˌskɛlətənaɪˈzeɪʃən/
Phonetic Spelling: skel-uh-tuh-ny-ZAY-shuhn

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4. phototoxicity
Pronunciation link: No confirmed link found
IPA: /ˌfoʊtoʊtɒˈsɪsɪti/
Phonetic Spelling: foh-toe-tocks-IS-i-tee

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5. photobleaching
Pronunciation link: No confirmed link found
IPA: /ˈfoʊtoʊˌbliːtʃɪŋ/
Phonetic Spelling: foh-toe-BLEE-ching
